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Effects of Cholinesterase Inhibitors on the Secretion of Beta-Amyloid Precursor Protein in Cell Cultures[a]

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Alzheimer's disease (AD) is characterized by depositions of the amyloid beta-peptide (Aβ) in the form of cerebrovascular amyloid and extracellular plaques.[1,2] Aβ of 39-43 amino acid residue is derived from a family of larger beta-amyloid precursor proteins (βAPP) of 695-770 amino acid residue.[1,2] A group of proteases generate carboxyl-truncated soluble derivatives of βAPP (sAPP) that are secreted in the conditioned medium of cell cultures and human plasma and in cerebrospinal fluid.[1] Different factors that regulate depositions of amyloid are central to understanding the cerebrovascular changes in AD. This disease is also marked by the dramatic loss of cholinergic neurons that project to the cortex and neurochemically by a reduction in presynaptic markers of the cholinergic system particularly in the areas of the brain related to memory and learning. In this report, we have investigated the possibility whether the processing of βAPP can be regulated by different cholinesterase inhibitors (ChE-Is), some of which have been reported to improve memory deficits and cognitive functions in some AD patients.[3,4] We have recently demonstrated that the metabolism of βAPP is regulated by tacrine.[5] Here we report a comparative study showing that the ChE-Is regulate the secretion of sAPP in a cell type–specific manner and that this effect may be independent of the anticholinesterase function.

MATERIALS AND METHODS

Materials

3,4-Diaminopyridine, metrifonate, physostigmine, and tacrine were bought from Sigma (MO). Fetal bovine serum (FBS), horse serum, and different media (DMEM, RPMI) were purchased from Gibco/BRL (MD). The rest of the chemicals were of molecular biology grade.

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Cells and Culture Conditions

Human neuroblastoma (SH-SY5Y), human glioblastoma (UA138), rat pheochromocytoma (PC12), and human fibroblast (HeLa) cells were obtained from American Type Culture Collection (ATCC) (MD), and cultured as described previously.[5] Untransfected cells were used throughout this study.

Antisera

The 22C11 clone (Boehringer Mannheim, IN) recognizes all mature forms of βAPP found in cell membranes as well as the carboxyl-truncated, soluble forms secreted into the conditioned media and the APP-like proteins (APLP). The epitope region was assigned to βAPP_{66-81} in the ectoplasmic cysteine-containing domain. A biotinylated secondary antibody, horse anti-mouse (Boehringer Mannheim), was also used. The details of the use of these antibodies have previously been described.[5]

Treatment of Cells with Drugs

1 to 1.5 × 10^7 of each cell type was cultured in its respective medium. Before adding drug, the cells were fed with media containing only 0.5% FBS (low serum). The cells were then incubated either in the absence or presence of a particular drug. Following incubation for 12–16 hours, the conditioned medium from each plate was collected and clarified at 800 *g* for 10 minutes. For the purpose of control experiments, an equivalent concentration of ethanol was used as a vehicle which was less than 0.1% in media. The following concentration of different drugs was used: 3,4-diaminopyridine (0.5 mM), metrifonate (0.5 mM), physostigmine (0.5 mM), and tacrine (0.15 mM).

Polyacrylamide Gel Electrophoresis and Immunoblotting

100 μl of conditioned media were separated on a 12% polyacrylamide gel containing SDS (SDS-PAGE). Immunoblot (western blot) analysis was performed using the avidin-biotinylated complex detection kit (Vector Laboratories, CA) as described previously.[5]

RESULTS

In this report we have compared the effects of four ChE-Is: 3,4-diaminopyridine, metrifonate, physostigmine, and tacrine on the secretion of sAPP in four cell types—human glioblastoma, neuroblastoma, and epithelial and rat chromaffin cells—using the immunoblotting technique. In a typical Western blot of conditioned media, sAPP were detected as 110–140-kDa bands which represent different isoforms and/or the post-translationally modified derivatives of βAPP. Secreted derivatives of sAPP generated by either the α- or β-secretase pathway are shorter in length than the fully matured βAPP forms observed intracellularly.

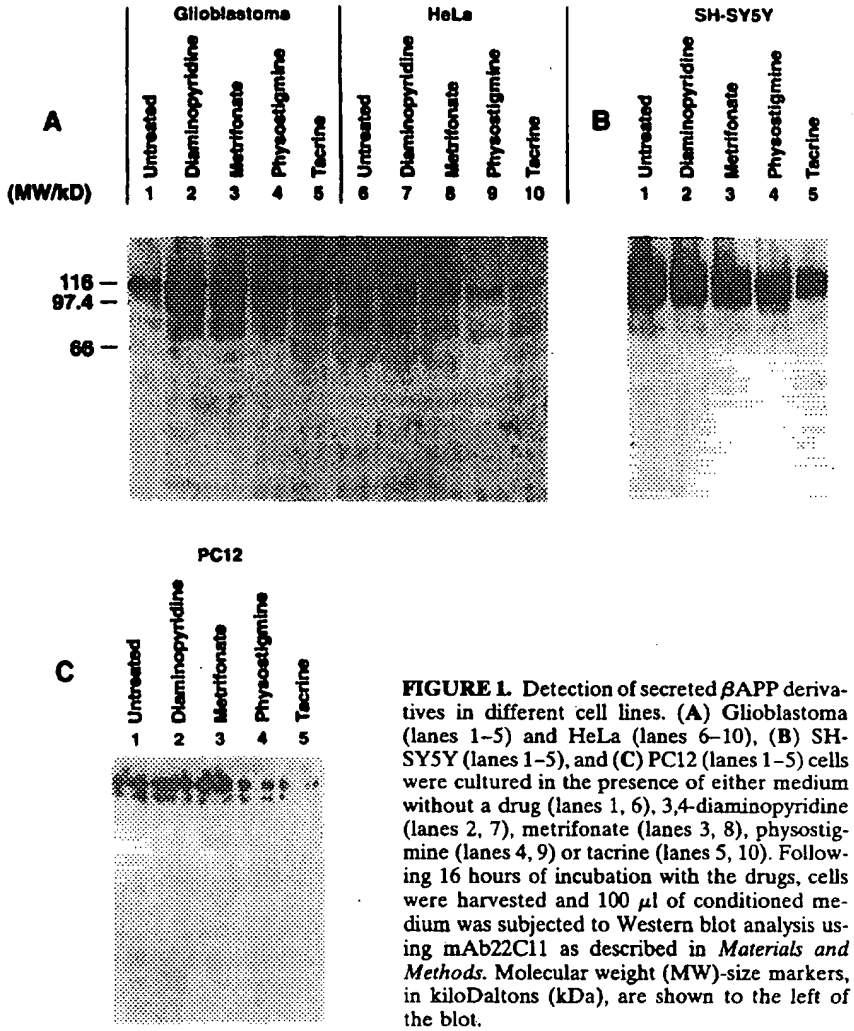

FIGURE 1. Detection of secreted βAPP derivatives in different cell lines. (**A**) Glioblastoma (lanes 1–5) and HeLa (lanes 6–10), (**B**) SH-SY5Y (lanes 1–5), and (**C**) PC12 (lanes 1–5) cells were cultured in the presence of either medium without a drug (lanes 1, 6), 3,4-diaminopyridine (lanes 2, 7), metrifonate (lanes 3, 8), physostigmine (lanes 4, 9) or tacrine (lanes 5, 10). Following 16 hours of incubation with the drugs, cells were harvested and 100 μl of conditioned medium was subjected to Western blot analysis using mAb22C11 as described in *Materials and Methods*. Molecular weight (MW)-size markers, in kiloDaltons (kDa), are shown to the left of the blot.

Effects of Cholinesterase Inhibitors on the Secretion of sAPP in Glioblastoma

As compared to untreated glioblastoma, the cells that were treated with either 3,4-diaminopyridine or metrifonate showed an increase in secretion of sAPP in the conditioned medium (FIG. 1A, lanes 1–3). Under the same conditions as those used to treat the cells with physostigmine, there was no significant change in secretion of sAPP, and with tacrine there was a decrease in the secretion of sAPP in the medium (lanes 4–5).

Effects of Cholinesterase Inhibitors on the Secretion of sAPP in HeLa Cells

As compared to the untreated HeLa cells, the cells that were treated with either 3,4-diaminopyridine or metrifonate displayed no significant change in levels of sAPP in the conditioned medium (lanes 6–8). Under the same conditions as those used in physostigmine treatment, there was a slight decrease in levels of sAPP, and with tacrine there was a sharp decrease in the secretion of sAPP in the medium (lanes 9–10).

Effects of Cholinesterase Inhibitors on the Secretion of sAPP in Neuroblastoma

As compared to the untreated neuroblastoma, the treatment of cells with either 3,4-diaminopyridine or metrifonate resulted in no change in levels of sAPP in the conditioned medium (FIG. 1B, lanes 1–3). Under the same conditions as those used when the cells were treated with physostigmine, there was a slight decrease in levels of sAPP, and with tacrine there was a sharp decrease in the secretion of sAPP in the medium (lanes 4–5).

Effects of Cholinesterase Inhibitors on the Secretion of sAPP in PC12 Cells

As compared to the untreated PC12 cells, treating cells with either 3,4-diaminopyridine, metrifonate, or physostigmine resulted in no change in secretion of sAPP in the conditioned medium (FIG. 1C, lanes 1–3). Under the same conditions when the cells were treated with tacrine there was a sharp decrease in secretion of sAPP in the medium (lanes 4–5).

DISCUSSION

The effect of 3,4-diaminopyridine and metrifonate increasing secretion of sAPP in glioblastoma is interesting. However, these two drugs as well as physostigmine showed no effect on the secretion of sAPP in the other cell types studied. We tested these drugs for anticholinesterase activity under the dose and conditions used here by using a modification of the Ellman technique.[6] All caused complete inhibition. Thus, although (1) physostigmine undergoes spontaneous hydrolysis to eseroline, leading to inactivation of its cholinesterase action, and (2) metrifonate undergoes spontaneous hydrolysis to the active cholinesterase inhibitor, dichloros, both occurred at a sufficient rate to produce anticholinesterase action in the present study. Although the treatment of cells with tacrine resulted in a decrease in levels of sAPP across cell lines, we consistently observed greater decreases in sAPP release in cell types of neuronal origin such as SH-SY5Y and PC12 cells. Immunoblots with KPI-specific antibodies suggested that at least two bands correspond to soluble derivatives from KPI-containing and KPI-lacking forms of sAPP, respectively (data not shown). To confirm that the proteins detected by mAb22C11 were sAPP, and not due to only a APP-related homologue such as APLP-2, we performed similar immunoblot analyses using mAb6E10, which is a human-specific monoclonal antibody raised against residue 1-16 of the Aβ sequence (Senetek, MO). The results were similar, indicating that the drug-mediated change was specific for sAPP. In a separate series of experiments we have tested phenserine, a 75-fold acetyl- vs.

butyrylcholinesterase-selective inhibitor,[7] in neuroblastoma cells. When the cells were treated with phenserine, we observed a reduction in the release of sAPP in the medium (data not shown).

The mechanisms by which ChE-Is regulate the secretion of sAPP are not fully understood. From the IC_{50} values, it is noted that these ChE-Is have some overlapping acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity at higher concentrations.[7] The effect of tacrine on the secretion of sAPP may not be due to its effect on the enzyme itself; rather it may be due to a lysosomotropic action because of its high pKa (~9.2). Moreover, even if cholinesterase inhibition is critical to this effect, there is preliminary evidence that tacrine and the other ChE-Is may bind to different sites on the enzyme and that these differences may influence their ability to regulate the secretion of sAPP. The difference in action of tacrine and physostigmine on the processing of βAPP in cell lines may also be due either to the former's selectivity for BChE over AChE or the instability of physostigmine in aqueous solution. However, the preliminary results from the experiments with phenserine suggest that the effects of the ChE-Is on sAPP release do not depend upon their selectivity for either AChE or BChE. Further experiments to determine the effects of these drugs on the levels of different forms of soluble Aβ and carboxyl terminal fragments of βAPP are in progress.

Our results indicate that as compared to the treatment of cells with tacrine, treating cells with other ChE-Is did not result in any uniform change in the secretion of sAPP in the conditioned medium from neuronal cell lines. This difference in action of metrifonate, physostigmine, phenserine, and tacrine on sAPP therefore may be independent of their anticholinesterase activities. For example tacrine was originally synthesized in 1945 as an antibacterial agent. In 1949, it was reported to be a morphine antagonist, and in 1953 was demonstrated to possess anticholinesterase activity. Recent reports have demonstrated cholinergic actions unrelated to acetylcholinesterase. Tacrine has been reported to stimulate acetylcholine release by blocking potassium channels, and to act directly on nicotinic and muscarinic receptors.[8] Tacrine was shown to have only a moderate effect on the decline of cognitive function in patients with AD.[3] In the cell culture system, tacrine at 0.15 mM specifically decreased the secretion of sAPP in the conditioned medium. At this concentration of tacrine, there is an almost complete inhibition of AChE activity. The concentration of tacrine required for cell culture is higher than the dosage of tacrine (160 mg/day) used in clinical trials. It is improper to compare the dosage used between cell culture studies and clinical trials, perhaps because of the difference (1) in the physiologically active conformation states of the enzyme and (2) in the actual local concentration of the drug. Our data demonstrate that the secretion of sAPP is affected by treating cells with tacrine and that clinical trials with a very high dosage of tacrine or the long-term treatment of AD patients with tacrine may be linked to altered proteolysis of βAPP. This is consistent with the previous hypothesis that tacrine may modulate the processing of βAPP by directly blocking the proteolytic activity associated with AChE.[5] Our results suggest that noncatalytic functions of cholinesterase inhibitors can potentially be utilized to alter the metabolism of βAPP, which might in turn affect the process of deposition of Aβ, a key component of the extracellular and vascular depositions of amyloid detected in Alzheimer's disease.

SUMMARY

One of the main characteristics of Alzheimer's disease (AD) is the cerebrovascular deposition of the amyloid β-peptide (Aβ), which is derived from a larger beta-

amyloid precursor protein (βAPP). The majority of βAPP is processed by either a secretory or lysosomal/endosomal pathway. Carboxyl-truncated soluble derivatives of βAPP (sAPP) are generated by the proteolytic processing of full-length βAPP by either α- or β-secretase enzyme. Our objective is to determine whether the processing of βAPP can be regulated by cholinesterase inhibitors, some of which were shown to produce a moderate improvement in memory and cognitive functions in patients with Alzheimer's disease. Here we have analyzed the levels of sAPP derivatives in cultured cells treated with different drugs by immunoblotting samples of conditioned media. The immunoreactive protein bands were developed by probing with the monoclonal antibody 22C11. Treating neuroblastoma, pheochromocytoma and fibroblast cells with high dose of either 3,4-diaminopyridine, metrifonate, or physostigmine did not inhibit the secretion of sAPP. Treating glioblastoma with either 3,4-diaminopyridine or metrifonate showed an increase in secretion of sAPP. However, treatment of cells with tacrine reduced release of sAPP in conditioned media of cell lines studied. The difference in action of metrifonate, physostigmine, and tacrine on βAPP is independent of their anticholinesterase activities. Our results suggest that noncatalytic functions of cholinesterase inhibitors can be utilized to alter the metabolism of βAPP, which might in turn affect the process of deposition of Aβ, a key component of the cerebrovascular amyloid detected in AD.

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